Inventory of Supplemental Information

Figures:

Movies:

Experimental Procedures

Supplementary Information

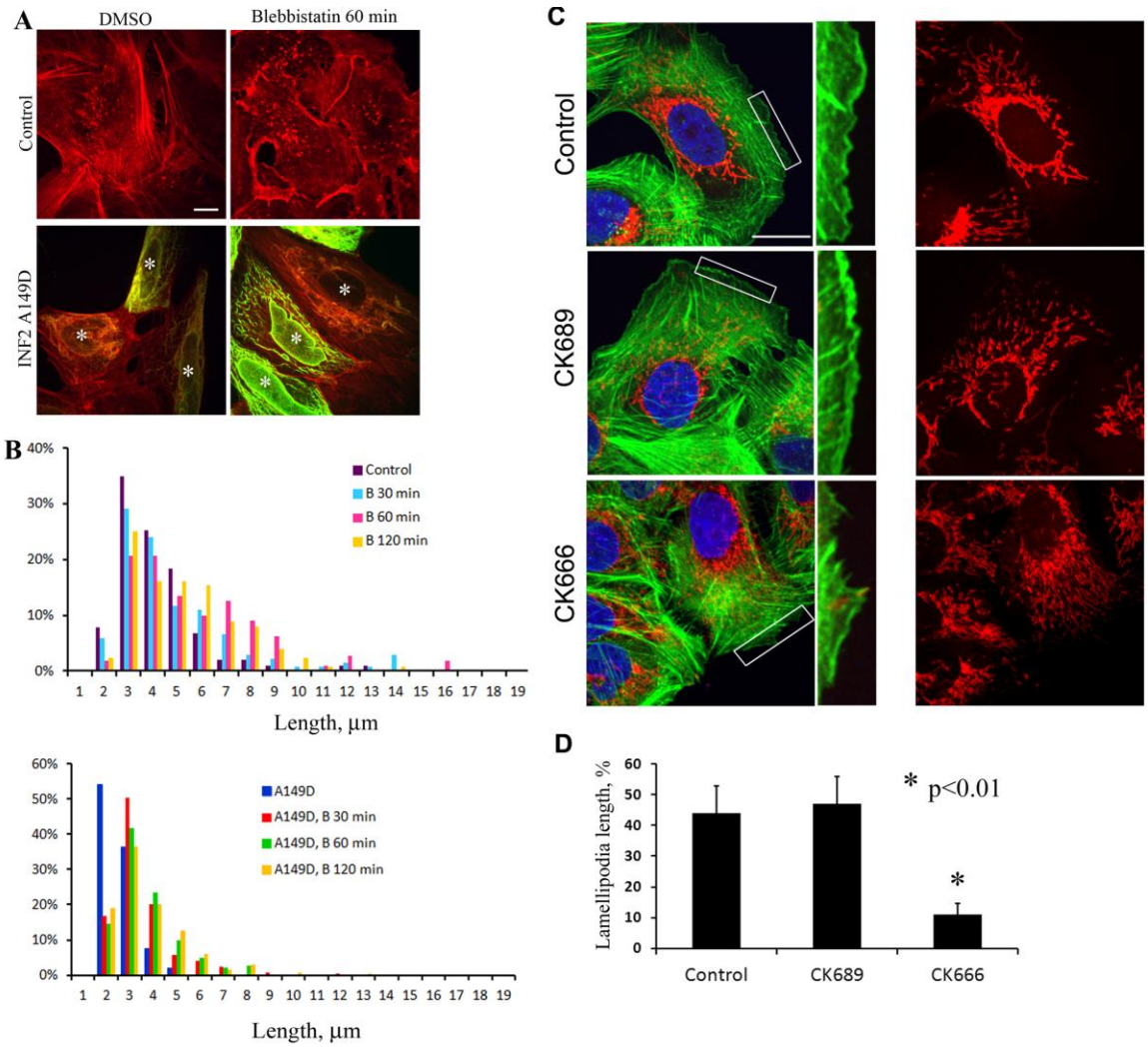

Figure S1. Blebbistatin treatment mitigates the effect of INF2 A149D on mitochondria size.

(A) Control U2OS cells or cells expressing GFP-INF2 A149D were treated with DMSO (left) or 50 µM blebbistatin (right). Actin (red) and INF2 A149D (green) staining for same panels as Figure 1. Asterisks indicate cells expressing GFP-INF2 A149D. Scale bar, 10 µm.

(B) Distribution of frequencies of mitochondrial length in control (top) or INF2 A149D expressing cells (bottom), treated with DMSO or 50 µM blebbistatin for indicated time points. Blebbistatin treatment significantly increases population of long mitochondria. N=103 to 344 mitochondria.

(C) Inhibition of Arp 2/3 complex inhibits lamellipodia but does not affect mitochondrial length. U2OS cells were untreated or treated with 200 µM of CK689 or CK666 for 1 h, and stained with fluorescein-phalloidin for actin filaments (green) and Mitotracker (red). Selected regions of cell leading edge show reduction of lamellipodia for CK666 but not for CK689 or control. Scale bar, 20 µm.

(D) Quantification of lamellipodia as a percent of total leading edge. N= 9 to 11 cells. Error bars, SEM.

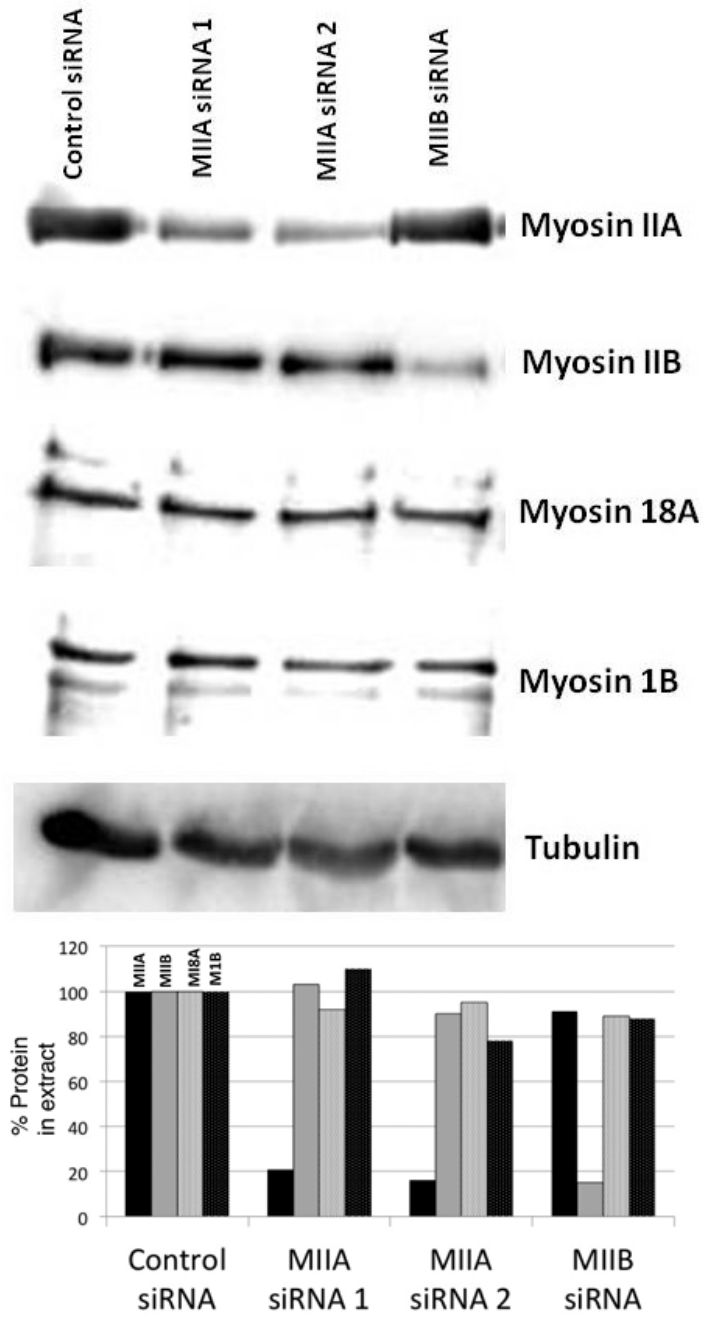

Figure S2. siRNA suppression of myosin IIA or myosin IIB.

Top: Western blots of U2OS lysates transfected with control siRNA or siRNAs for myosin IIA or myosin IIB, as indicated above blots. Blots probed with antibodies for myosins or tubulin, as indicated at right of blots.

Bottom: Quantification of myosin bands by densitometry, normalized to tubulin control bands. In all cases, antibody signal was tested for linearity with varying extract load.

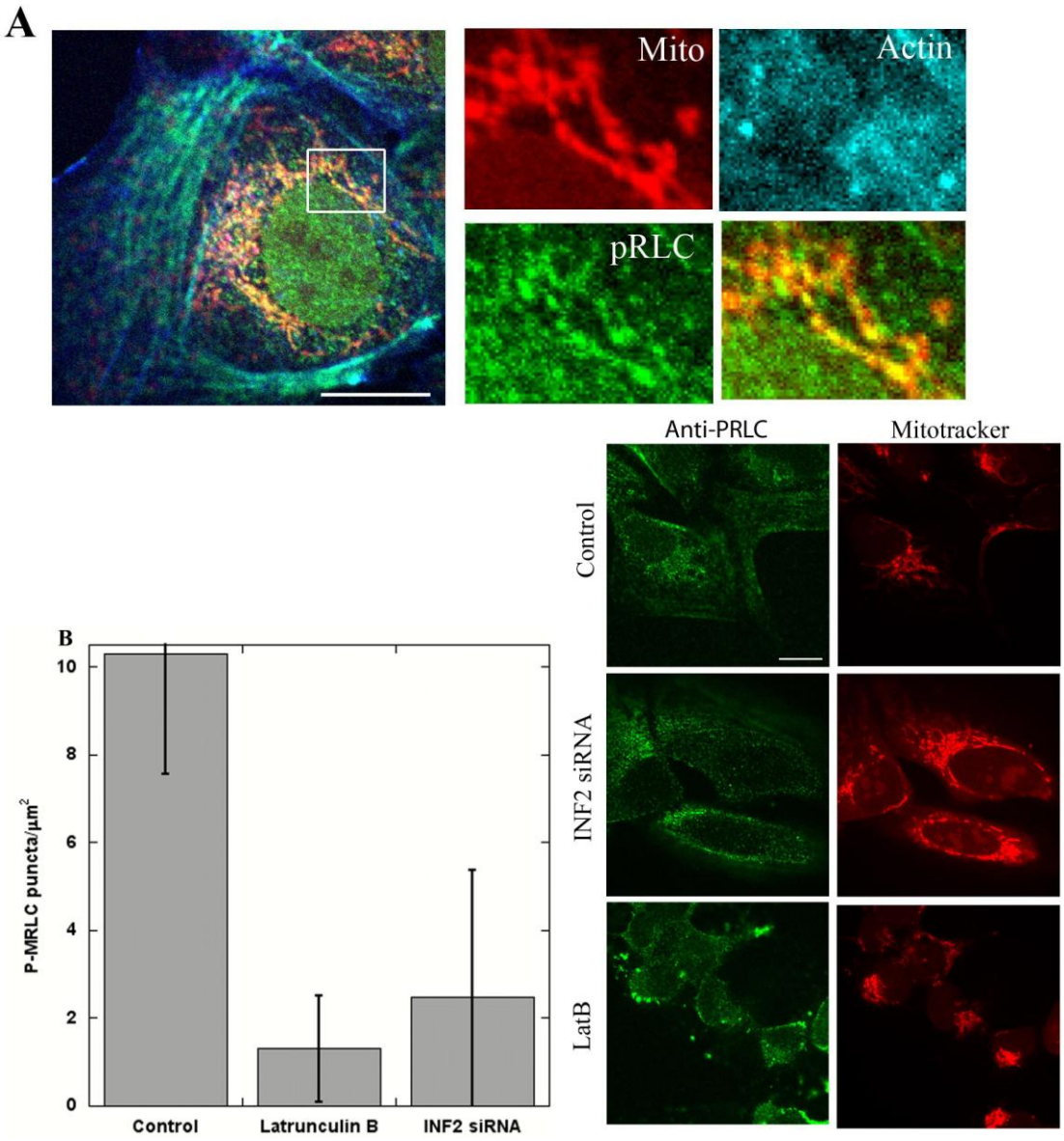

Figure S3. Active myosin accumulates on apical mitochondria in an actin- and INF2-dependent manner.

(A) Left: maximum intensity projection of a confocal Z-stack of a U2OS cell labeled with mitotracker, fixed and stained with anti-pMRLC and alexa405-phalloidin. Scale bar, 10 μm. Right: close-up of single Z-section of apical cell region, from the boxed region.

(B) Quantification of P-MRLC enrichment at apical mitochondria in control cells or cells treated with 0.5 μM LatB for 60 min or with siRNA for INF2 for 72 hrs, from images similar to those in Figure 3D.

(C) Single fluorescence images for the experiment described in Figure 3B. U2OS cells were treated with INF2 siRNA for 72 hours, or with 0.5 μM LatB for 60 min, then mitotracker stained, fixed, and stained for anti-P-MRLC. Scale bar, 20 μm.

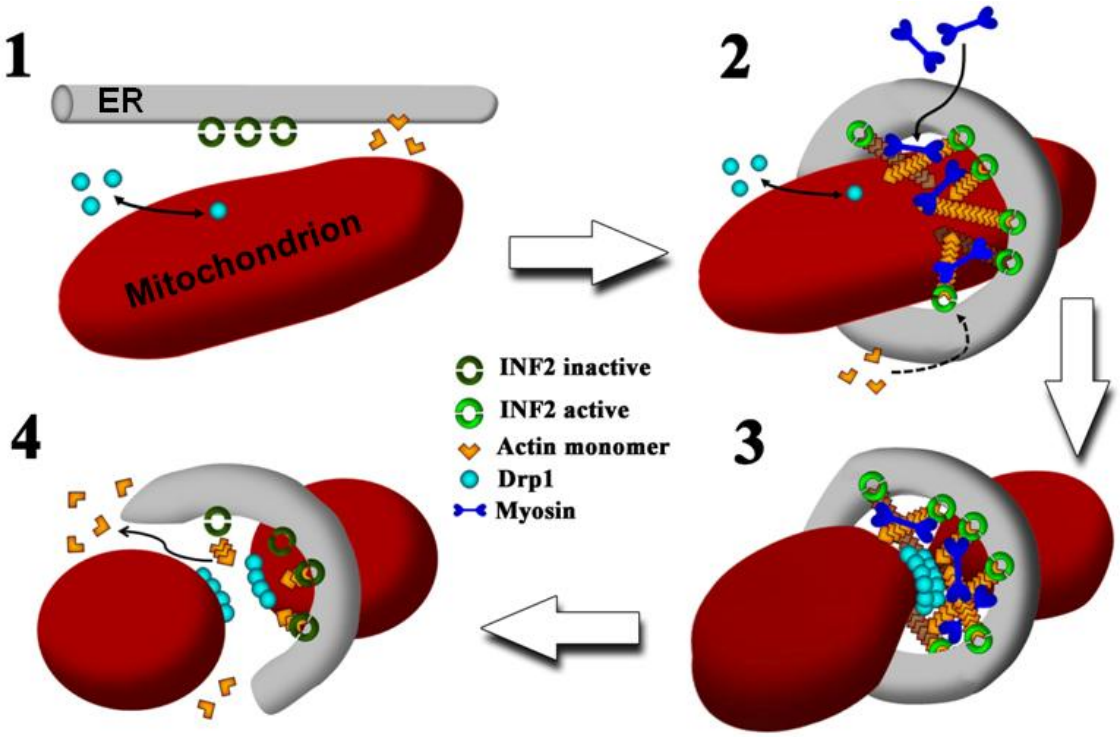

Figure S4: A model for myosin II function during mitochondrial fission.

Step 1: ER (grey) and mitochondrion (red) are not in contact. ER-bound INF2 is inactive. Drp1 is not tightly associated with the mitochondrion.

Step 2: ER-mitochondrial interaction activates INF2, which assembles actin filaments at the interface between the two organelles. Myosin II is recruited to these filaments, and myosin II motor activity causes initial constriction of the mitochondrion.

Step 3: Drp1 is recruited to this constriction site, assembles into a ring, and Drp1 GTPase activity causes constriction of this ring to drive mitochondrial fission.

Step 4: Upon fission, ER detaches from mitochondrion. INF2 inactivates, actin depolymerizes.

This is but one possible model from the available data. We do not rule out other orientations of actin filaments and myosin that could produce similar constrictive forces.

Movie S1. Mitochondrial fission event with accumulation of myosin II in constriction site. U2OS cell expressing mito-red and GFP-MIIA were analyzed in time lapse movie for 2.5 min with 4 sec interval. Single confocal slice is shown. Constriction site on mitochondrion starts to be visible at ~ 76 sec, and fission happens at 108 sec. See also Figure 3C.

Supplemental Experimental Procedures

Plasmids and siRNA oligonucleotides

Mito-BFP was a gift from Gia Voeltz (University of Colorado). Mito-dsRed was a gift from Ekta Chhabra (Massachusetts General Hospital).

Oligonucleotides for human myosins II siRNA were synthesized by IDT Oligo against following target sequences:

5'-GCCACGCCCAGAAGAACGAGAAUGC-3' (Myosin IIA siRNA#1), and 5'-GCAAGCUGCCGAUAAGUAUCUCUAT-3' (Myosin IIA siRNA#2), 5'-ACACUCCAUAAGAACAAUGCCCUTA-3' (Myosin IIB siRNA), 5'-GGAUCAACCUGGAGAUCAUCCGC-3' (INF2 siRNA). As a control, Silencer Negative Control #1 siRNA (Ambion) was used.

Cell culture, transfections and drug treatments

U2OS cell lines were grown in DMEM (Invitrogen) supplemented with 10% calf serum (Atlanta Biologicals). Cells were seeded at $2x10^5$ cells per well on a 6-well dish ~16 hours prior to transfection. Plasmid transfections were performed in OPTI-MEM media (Invitrogen) with 2 μL Lipofectamine 2000 (Invitrogen) per well. The following amounts of DNA were transfected per well: 30 ng mito-dsRed and mito-BFP; 60 ng ER-green; 50 ng for INF2. For RNAi transfections, cells were plated on 6 well plates at 30-40% density for ~16 hrs, then 2 μl RNAmax (Invitrogen) and 63 pg of siRNA were used per well. Cells were analyzed 24 hr and 72-80 hr post-transfection for DNA and RNAi respectively. When needed, cells were treated with MitoTracker Red CMXRos (Invitrogen) at 100 nM in DMEM for 20 min prior to fixation. For chemical inhibitor treatments, cells were incubated with medium containing 50 μM blebbistatin (Sigma-Aldrich), 200 μM CK666 or CK689 (Calbiochem), or 0.5 μM Latrunculin B (Calbiochem) for the indicated times, with DMSO used as the negative control.

Antibodies

Polyclonal nonmuscle myosin IIA and myosin IIB antibodies (Cell Signaling) were used at 1:1000 for western blotting. Polyclonal antibodies against pRLC (phospho-myosin regulatory light chain 2, Ser19, Cell Signaling) were used 1:10 dilution for immunostaining. Anti-tubulin (DM1-α, Sigma) was used at 1:10,000 dilution for western. Drp1 was detected using a rabbit monoclonal antibody (Cell Signaling) at 1:50 dilution for immunostaining. Secondary antibodies used were TexasRed, Cy5 or Fluorescein conjugated anti-rabbit IgG, (Jackson Immunoresearch and Vector Laboratories, respectively), and used at 1:300 dilution.

Immunofluorescence Microscopy

Cells were fixed with 4% formaldehyde (Electron Microscopy Sciences, PA) in phosphate-buffered saline (PBS) for 30 min at room temperature. After washing with PBS, cells were permeabilized with 0.1% Triton X-100 in PBS for 15 min. Cells were then washed with PBS, blocked with 0.5% BSA in PBS for 1 hr, and incubated with primary antibodies in PBS for 1 hr at room temperature. After washing with PBS, secondary antibodies were applied for 1hr at room temperature. When needed, 500 nM AlexaFluor450-phalloidin (Invitrogen) or 100 nM TRITC-phalloidin (Sigma/Aldrich), and 10 μM 4,6-diamidino-2-

phenylindole (DAPI) were added to secondary antibody solution. Samples were mount on polyvinyl alcohol-DABCO.

Confocal microscopy for fixed and live cells

Imaging of live and fixed cells was performed using a spinning disk confocal system. For live imaging, cells grown on 18 mm coverslips were mounted into Rose chambers, then onto a Wave FX spinning disk confocal microscope (Quorum Technologies, Inc., Guelph, Canada, on a Nikon Eclipse Ti microscope) with Bionomic Controller (20/20 Technology, Inc) temperature-controlled stage set to 37°C. After equilibrating to temperature for 10 min, cells were imaged with the 60x 1.4 NA Plan Apo objective (Nikon) using the 403 nm and 450/50 filter for BFP, 491 nm laser and 525/20 filter for GFP, and the 561 nm laser and 593/40 filter for mRFP. For fixed cells, 6-8 z-stacks of 0.2 μm were collected for each color. Maximum intensity projections from best focus Z slices were assembled using Metamorph software and processed using Nikon Elements and Photoshop CS (Adobe, San Jose, CA). For live cells, a single z slice was collected at 4 sec intervals for 10 min.

Measurements and image analysis

To measure mitochondrial length, maximum intensity projections of z-series with 0.2 μm increments for red channel (Mitotracker or mito-dsRed) were created. The flat regions of cells with clearly resolved mitochondria were selected, and 25-30 mitochondria per cell were measured using the line tool in Nikon Elements software. Drp1 puncta were counted on fixed cells labeled with anti-Drp1 antibody. Only mitochondria-associated puncta were quantified, and their association with mitochondria was verified on consecutive z-planes. To assess P-MRLC localization at sites of apical mitochondria, a representative confocal slice at least 1.4 microns from the ventral surface was chosen, and a 5 x 5 micron box within the region containing apical mitochondria was made. The number of P-MRLC puncta within this box was counted manually, with a positive punctum defined as a region of < 5 pixels in either dimension whose intensity was clearly above background. Based on these criteria, linear P-MRLC-containing structures (possibly dorsal stress fibers) were excluded. Statistic analysis was performed in Excel (Microsoft), data presented as mean ± standard error from at least two experiments. For frequencies of distribution, mitochondrial lengths pooled from two experiments were binned from 0 to 34 μm with 2 μm increment, and percentage value of each category was plotted using Excel. Unpaired Student's t-test was used to compare values with $p<0.01$ considered significant.

Western blotting

To prepare samples for SDS-PAGE, cells were grown on 6 well plate and transfected with siRNAs for 72-96 hrs, trypsinized, washed with PBS and resuspended 50 μL PBS. 50 μL was mixed with 34 μL of 10% SDS and 1 μL of 1 M DTT, boiled 5 minutes, cooled to 23°C, then 17 μl of 300 mM of freshly made NEM in water was added. Just before SDS-PAGE, the protein sample was mixed 1:1 with 2xDB (250 mM Tris-HCl pH 6.8, 2 mM EDTA, 20% glycerol, 0.8% SDS, 0.02% bromophenol blue, 1000 mM NaCl, 4 M urea). Proteins were separated by 7.5% SDS-PAGE and transferred to a PVDF membrane (polyvinylidine difluoride membrane, Millipore). The membrane was blocked with TBS-T (20 mM Tris-HCl, pH 7.6, 136 mM NaCl, and 0.1% Tween-20) containing 3% BSA (Research Organics) for 1 hour, then incubated with the primary antibody solution at 4°C overnight. After washing

with TBS-T, the membrane was incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (Bio-Rad) for 1 hour at room temperature. Signals were detected by Chemiluminescence (Pierce). Western bands were quantified using Image J, after background subtraction from another region of the same lane on the blot. Multiple dilutions of cell extract were analyzed to ensure that the Western signal was linear within the analyzed range for each protein probed.

Supplemental Movie and Spreadsheet

Click here to download Supplemental Movie and Spreadsheet: Movie S1.avi